roX2 CHART Protocol

To accompany:
Simon et al. “The Genomic Binding Sites of a Non-Coding RNA.”
December 2011

This document provides a detailed protocol for CHART enrichment of roX2 to determine its genomic binding sites. The sections included in this protocol are:

SECTION 1. PREPARING S2 CELL NUCLEI FOR CHART
SECTION 2. DETERMINING REGIONS of RNase H SENSITIVITY
SECTION 3. DESIGN AND SYNTHESIS OF C-OLIGOS FOR CHART
SECTION 4. CHART EXTRACT PREPARATION
SECTION 5. CHART ENRICHMENT
SECTION 6. PREPARATION OF DNA FROM CHART ENRICHMENT
SECTION 7. LIBRARY CONSTRUCTION AND SEQUENCING
APPENDIX 1. ANALYSIS OF CHART RNA ENRICHMENT BY qPCR
APPENDIX 2. ANALYSIS OF CHART DNA ENRICHMENT BY qPCR
SOLUTIONS AND REAGENTS.

SECTION 1. PREPARING S2 CELL NUCLEI FOR CHART

Materials

CCM3 medium (Hyclone).
PBS
Formaldehyde (16% w/v, 10 mL ampule, Thermo Scientific, cat. 28908)
Sucrose Buffer
Glycerol Buffer
Glass dounce homogenizer with tight pestal (15 mL)

Protocol

1. Grow Drosophila S2 cells in shaker flasks in serum-free CCM3 medium.

2. Harvest by centrifugation (~10^{10} cells, 500g, 15 min), rinse once with cold PBS and resuspended in PBS (200 mL).

3. Add formaldehyde to 1% final concentration and allow the suspension to rotate end-over-end (10 min, rt).

4. Capture the cells by centrifugation, rinse three times with cold PBS and use immediately or aliquot (1x10^9 cells/aliquot), flash freeze (N_2) and store at -80°C.

5. Resuspend the pellet in Sucrose Buffer (4 mL, on ice).
6. Transfer the suspension to an ice-cold dounce homogenizer. Dounce ten times with a tight pestle. Wait five minutes. Then dounce ten more times.

7. Add 4 mL of Glycerol Buffer to a 15 mL conical tube. Then add 4 mL of Glycerol Buffer to the mixture in the dounce homogenizer and mix by pipetting up and down. Carefully layer the cell debris on top of the Glycerol Buffer in the 15 mL conical tube.

8. Centrifuge the tube (1000g for 10 min, 4°C) to pellet the nuclei.

9. Remove the supernatant using a pipette, taking care to pull off the upper layer with minimal mixing.

10. Repeat steps 2-6 one additional time (two times total).

   This pellet of enriched nuclei can either be carried directly into the RNase H mapping protocol (SECTION 2), or further crosslinked and used for CHART enrichment (SECTION 4).

SECTION 2. DETERMINING REGIONS of RNase H SENSITIVITY

Materials

- Nuclei pellet (from SECTION 1)
- Nuclei Wash Buffer
- Sonication Buffer
- Covaris S2 instrument
- RNase H (NEB, 5U/µL, cat. M0297L)
- SUPERasIN (Ambion)
- 20-mer oligonucleotides that tile roX2 RNA
- DNase RQ1, Promega
- Proteinase K (20 mg/mL solution)
- PureLin Micro-to-Midi Total RNA Purification System (Invitrogen, cat. 12183-018)
- Nanodrop spectrophotometer
- SuperScript VILO cDNA Synthesis Kit (Invitrogen, cat. 11754-050)
- ABI 7500 RT-PCR instrument
- iTaq SYBR Green Supermix with ROX (BIO-RAD)
- Appropriate qPCR primer sets (See PRIMERS)

Protocol

1. Resuspend the nuclei in Nuclei Wash Buffer (5 mL, on ice).

2. Centrifuge the tube (1000g, 10 min, 4°C) to pellet the nuclei.
3. Repeat steps 1 & 2 one more time (two rinses total).

4. Resuspend pellet in 3 mL of Sonication Buffer and centrifuge as in step 2.

5. Resuspend the pellet to 3 mL final volume (~1.5 mL added buffer) of Sonication Buffer.

   While the Sonication Buffer listed here was successfully used for mapping of roX2 RNA in S2 cells, subsequent experiments have revealed that up to 4-fold lower detergent concentrations in the buffer lead to better performance of this assay and do not appear to compromise solubility.

6. Process the nuclei using a Covaris instrument (30 min. program, 10% duty cycle, intensity of 5, 4°C) to make soluble the chromatin.

   This assay has been successful using extract solubilized by different means, including Bransen sonication, and with average sheer sizes ranging from 200bp-5kb. It is likely that any instrument successfully used for ChIP experiments can be applied successfully (so long as it does not lead to RNase contamination of the extract, which is one advantage of using a non-invasive instrument like Covaris).

7. Separate the extract into four 1.7 mL tubes and clear the extract by centrifugation (16.1kg, 10 min., rt).

8. Separate the cleared extract into aliquots and continue to step 9 immediately or flash freeze (N₂) and store at -80°C.

9. Set up a master mix (e.g., 36x master mix) of the following reagents:

   - 10 µL cleared extract (360 µL)
   - 0.03 µL MgCl₂ (1 M stock) (1.1 µL)
   - 0.1 µL DTT (1M stock) (3.6 µL)
   - 1 µL RNase H (36 µL)
   - 0.5 µL SUPERasIN (20 u/µL) (18 µL)

10. In 8-strips of PCR tubes, add to each tube 10 µL master mix.

11. Add 1 µL of DNA oligo (100 pmol/µL stock) to each tube except for two controls where water should be used instead of a DNA oligonucleotide.

12. Mix by pipetting up and down 20 times. Concentrate the liquid in the tubes by quick (~5 sec.) centrifugation.

13. Incubate in a PCR machine at 30°C for 30 min.
A range of temperatures (30-37°C) and times (30 min-1hr) have been successfully employed.

14. Quick spin the tubes and add 1 μL of DNase master mix:

\[
\begin{align*}
1\mu L & \text{ per reaction RQ1 DNase (40 }\mu L) \\
0.1\mu L & \text{ per reaction of 60 mM CaCl}_2 \text{ (made from 6 }\mu L 1 \text{ M stock into 94 }\mu L \text{ ddH}_2\text{O) (4 }\mu L)
\end{align*}
\]

15. Incubate 30°C for 10 min.

16. Quick spin and quench by adding 2 μL of freshly made quenching buffer into the cap, close, quick spin, mix and quick spin once more.

\[
\begin{align*}
20\mu L & \text{ 0.5 M EDTA} \\
20\mu L & \text{ 1M Tris pH 7.2} \\
20\mu L & \text{ 20 mg/mL Proteinase K (added immediately before use)} \\
20\mu L & \text{ 10% SDS}
\end{align*}
\]

17. Incubate in a PCR thermocycler for 60 min at 55°C; then 30 min at 65°C.

18. Quick spin. Purify RNA using PureLink RNA isolation kit according to the manufacture’s directions. Include extra-on column DNase step. Elute into 30 μL ddH$_2$O.

*Other companies’ products have also been successfully used. Also note that the earlier DNase treatment (Step 14) is prior to crosslink reversal (Step 17), and therefore an second DNase treatment is included to remove DNA that was protected by crosslinking.*

19. Determine the approximate concentration of RNA using a Nanodrop spectrophotometer.

*This step is for quality control to ensure the RNA was not lost during handling. Usually the yield is between ~100-200 ng/μL.*

20. Set up reverse transcription reactions as follows:

\[
\begin{align*}
2\mu L & \text{ 5X VILO master mix} \\
7\mu L & \text{ RNA solution from step 18.} \\
1\mu L & \text{ VILO RT enzyme (include one RT-)}
\end{align*}
\]

21. Incubate as instructed (25°C 10 min.; 42°C 60 min.; 85°C 5 min.; 4°C forever).

22. Dilute the RT reactions with ddH$_2$O (10μL).
23. Analyze by qPCR using an ABI 7500 RT-PCR instrument and iTaq SYBR Green Supermix with ROX (the dye, unrelated to \textit{roX2}).

- 12.5 µL Supermix (need about 1.25 mL/plate)
- 10.5 µL of primer mix (3 µL ea. primer into 420 µL H2O)
- 2 µL of RT reaction (use multichannel)

(94°C 5 min. 40 cycles of [94°C 30”, 52°C 30”, 72°C 1”]).

For each RNase $H$ reaction, analyze using at least three primer sets: (1) a primer set that amplifies a region of the target cDNA that includes the oligo probe, (2) a control primer set for an unrelated RNA (e.g., \textit{Act5C} transcript) to normalize input levels and (3) a control primer set outside the putative region of sensitivity but part of the target cDNA.

24. Analyze results with the following formula:

$$\text{RNase H Sensitivity} = \left( \frac{C_{r_{\text{oligo}}} - C_{r_{\text{no oligo}}}}{C_{r_{\text{oligo}}} - C_{r_{\text{no oligo}}}} \right)$$

The locations of the peaks in sensitivity are robust, but the numerical sensitivities vary. This is acceptable because only the relative (and not the absolute) sensitivity is important. The efficiencies for each primer set can either be determined experimentally (as in Simon et al., fig S1A) or approximated as ~2.

\section*{SECTION 3. DESIGN AND SYNTHESIS OF C-OLIGOS}

\textit{NOTE:} For biotin-eluted CHART, the oligonucleotides were synthesized in-house as described below to reduce the high costs associated with the modifications (i.e., multiple linkers and desthiobiotin). For RNase $H$-eluted CHART, we found that a single C18-spacer was sufficient for enrichment when combined with 3’-biotin-TEG, and therefore we ordered these oligos commercially (IDT) with the design [OLIGO-SEQ]-L-TEG-biotin. Therefore this section is optional.

\textbf{Materials}

- Expidite DNA synthesizer
- Traditional DNA Phosphoramidite building blocks
- CPG resin pre-charged with desthiobiotin TEG (Glen Research, cat. 20-2952-41)
- C18-spacer phosphoramidite (Glen Research, cat. 10-1918-02)
- PolyPak II cartridges (Glen Research, 60-3100-10)
- Lyophilizer

\textbf{Protocol}
1. Analyze the peaks from RNase H mapping, focusing on regions with two or more oligos inducing specificity. Optimize a 24-25 nt sequence by BLAST for specificity in the genome and against off-target RNAs.

2. Use these sequences to synthesize oligonucleotides of the form: [OLIGO SEQ]-LLLLL-DSB, where L represents the C18-spacer, and DSB is desthiobiotin TEG. Performing the synthesis on a one µmol scale provides enough C-oligo for tens-to-hundreds of experiments.

3. Leave the final DMT protecting group on, and purify with PolyPack II cartridges according to the manufacture’s directions.

4. Lyophilize the product nucleotides and resuspend in 400 µL of EB (10 mM Tris pH 8.0). Analyze by 20% denaturing urea-PAGE. Generally the products are of sufficient purity that no further purification is necessary.

5. Use the OD\textsubscript{260} to determine the concentration of the CHART C-oligos in conjunction with their calculated extinction coefficient (e.g., using http://www.ambion.com/techlib/misc/oligo_calculator.html)

6. Make working dilutions of the C-oligo cocktails at 300 pmol/µL each oligo, (e.g., R2.1, R2.2, R2.3).

**SECTION 4. CHART EXTRACT PREPARATION.**

**Materials**

- Nuclei pellet from 1x10\textsuperscript{9} cells (SECTION 1)
- Wash Buffer 100 (WB100)
- Formaldehyde (16% w/v, 10 mL ampules, Thermo Scientific, cat. 28908)
- 1xPBS (pH 7.4)
- SUPERasIN (20 u/µL, Ambion, AM2696)
- Roche complete EDTA-free protease inhibitor cocktail

**Protocol**

1. Thaw a pellet of nuclei from **SECTION 1** on ice.

2. Rinse the pellet twice with PBS (10 mL) using centrifugation (1000g,10 min) to capture the nuclei between each rinse. Use the rinses to transfer the nuclei to a 50 mL conical.

3. Resuspend in PBS (40 mL) and add formaldehyde (entire 10 mL ampule of 16%). Rotate the tube for 30 min. at room temperature.
4. Centrifuge (1000g, 10 min, 4°C) to collect the nuclei and resuspend in 50 mL of PBS.

5. Centrifuge (1000g, 5 min, 4°C) to collect the nuclei and transfer to a 15 mL conical tube using two times 5 mL of PBS (10 mL total).

6. Centrifuge (1000g, 5 min, 4°C) to collect the nuclei and rinse twice with WB100.

7. Resuspend the nuclei to at 3 mL final volume of WB100 supplemented with SUPERasIN (20 u/µL final) and protease inhibitors.

8. Sonicate the nuclei at power level 5-6 (holding the output between 30-40W) for 10 min total process time (15” on, 45” off) in ice bath.

   We have also had success using Covaris to shear and solubilize the chromatin. These conditions should be determined empirically.

9. Separate into six 1.7 mL tubes and clear the extract by centrifugation (16,100g, 20 min at 4°C).

10. Aliquot the cleared extract (250-500 µL aliquots) and either continue directly to SECTION 5, or flash freeze (N2) and store at -80°C.

SECTION 5. CHART ENRICHMENT

Materials

- CHART extract (SECTION 4)
- SUPERasIN (20 u/µL, Ambion, cat. AM2696)
- Roche complete EDTA-free protease inhibitor cocktail
- Denaturant Buffer
- 2X Hybridization Buffer
- Ultralink Streptavidin Resin (Pierce, cat. 53117)
- Pierce spin cups (Pierce, cat. 69705)
- Hybridization oven
- MyOne Dynabeads C1 (Invitrogen, cat. 650.02)
- Dynal magnets for 1.7 mL and 15 mL tubes.
- Wash Buffer 250 (WB250)
- Biotin solution (50 mM, Invitrogen, cat. B20656)

Protocol

1. Thaw 500 µL of extract from SECTION 4.
2. Supplement the extract with:

   - 10 µL SUPERasIN
   - 5 µL DTT (1M)
   - 5 µL 100X protease inhibitors

3. Add 250 µL of Denaturant Buffer.

4. Add 750 µL of 2X Hybridization Buffer.

5. Pre-rinse two times 50 µL of Ultralink SAV resin two times each with 300 µL ddH₂O in two spin cups, spinning each time at 1000g for 0.5 min.

   *While steps 5-9 were included in our initial experiments, we have found they can be omitted without loss to enrichment of roX2 targets.*

6. Cap the bottom of the cups and add the extract from step 4, screwing the top caps on tightly.

7. Heat to 55°C in hybridization oven for 5 min.

8. Rotate end over end at room temperature for at least 1h.

9. Spin out extract and combine into one 1.7 mL tube.

10. For each CHART experiment, use ~400 µL, which leaves enough for the roX2 CHART, the sense control, and a no-oligo control (from which the supernatant can be considered input). Add 54 pmol (2.7 µL of the 20 µM CHART oligo cocktail section 3) for every 100 µL of extract (*i.e.*, 10.8 µL/400 µL extract). Mix thoroughly with a pipette.

   *Depending on the oligo cocktail, we have found that there is room for optimization of the concentrations of both individual C-oligos in the cocktail, and also the absolute concentration of C-oligos (usually we use 10-50 µM stocks at the volumes listed above).*

11. Separate into 75 µL aliquots in a PCR plate.

12. Run the following temperature cycle: 55°C 20'; 37°C 10'; 45°C 1h; 37°C 30'; rt forever.

   *Omitting this hybridization step reduced hybridization-induced artifacts.*

   *Instead we incubate overnight at room temperature (12-24h).*

13. Pool into 1.7 mL tubes and centrifuge to clear (16,100g, 20 min, rt).
15. Pre-rinse 150 µL MyOne Dynabeads with two times 500 µL ddH₂O using the magnetic stand to capture the beads in between rinses.

16. Resuspend beads in 100 µL ddH₂O and then add 50 µL Denaturant Buffer.

17. Add the cleared extract from ddH₂O and then add 50 µL Denaturant Buffer.

18. Capture the beads and save the supernatant from the no-oligo control for later analysis.

> The supernatant from a no-oligo control makes for a good input sample since it controls for any composition changes during handling of the samples.

19. Use 500 µL of WB250 to transfer the beads to a fresh 15 mL conical tube pre-charged with 5 mL of WB250. Capture with the beads with a Dynal magnet and wash three more times, completely resuspending the beads with gentle inversion between each mix (for four total washes in the 15 mL tubes).

For biotin-eluted CHART:

20a. Use 2x500 µL of WB250 to transfer the bead mixture to fresh 1.7 mL tubes.

21a. Capture the beads, remove the majority of the supernatant, centrifuge the tubes briefly (1000g, 5 sec), replace the tubes in the magnet and remove the residual liquid.

22a. Remove the tubes from the magnet and add 100 µL freshly made Elution Buffer.

23a. Incubate with shaking (1200 rpm) for 1h at rt.

For RNase H-eluted CHART:

20b. Use 2x500 µL of HEB to transfer the bead mixture to fresh 1.7 mL tubes.

21a. Capture the beads, remove the majority of the supernatant, centrifuge the tubes briefly (1000g, 5 sec), replace the tubes in the magnet and remove the residual liquid.

22a. Remove the tubes from the magnet and add 100 µL freshly made HEB, resuspending the bead by pipette.

23a. Add 2 µL RNase H and incubate at room with gentle shaking (800 rpm) for 10 min at rt.
24. Centrifuge the tubes briefly (1000g, 5 sec), and capture the beads.

25. Transfer the supernatant to a fresh tube and either process immediately or flash freeze (N$_2$) and store at -80°C.

**SECTION 6. PREPARATION OF DNA FROM CHART ENRICHMENT**

**Materials**

- CHART enriched eluant (SECTION 5)
- Proteinase K (20 mg/mL stock, molecular biology grade)
- Nucleic Acid XLR Buffer
- Phaselock tubes
- CHCl$_3$ (Fluka, cat. 25668)
- GlycoBlue (Ambion, AM9515, 15 mg/mL)
- Kimwipes
- Covaris MicroTube (6 x 16mm) AFA Fiber with Snap-Cap round bottom glass tube (cat. 520045)
- Phenol:CHCl$_3$:isoamyl alcohol 25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma, cat. P3803)

**Protocol**

1. To 100 µL of the eluant, add 25 µL Nucleic Acid XLR Buffer. Include additional tubes for analysis of the input.

2. Heat to 55°C for 1h and then to 65°C for 30 min.

3. Dilute 100 µL into 100 µL of ddH$_2$O in a 1.5 mL phaselock tube and 200 µL of Phenol:CHCl$_3$:isoamyl alcohol. Save 25 µL for analysis of the enriched RNA (APPENDIX 2).

4. Shake vigorously and centrifuge (12,000g, 5 min).

5. Rinse the aqueous layer twice with 100 µL CHCl$_3$.

6. Transfer the aqueous solution to a fresh tube and add 10 µL NaOAc (3M, pH 5.5) and 1 µL GlycoBlue. Then add 500 µL EtOH, vortex and incubate overnight at -20°C.

7. Pellet the nucleic acids by centrifugation (16.1kg, 20 min., 4°C).

8. Carefully remove the supernatant and rinse the pellet with 500 µL of 70% EtOH.
For long-term storage, keep pellet in the 70% EtOH rinse at -80°C.

9. Remove all the liquid, air dry for 5 min. at room temperature with the tube covered by a Kimwipe.

10. Resuspend the pellet in 100 µL of Tris buffer (10 mM, pH 8.0).

11. Transfer the liquid to a MicroTube for Covaris.

12. To reduce the average fragment size to 200-500 bp, process the tube by Covaris under the following conditions.

   DUTY CYCLE: 5%
   INTENSITY: 5
   CYCLES/BURST: 200
   TIME: 60 sec. (4 min program)
   BATH TEMP 4°C

13. Use this sheered material directly for library construction (SECTION 7).

SECTION 7. LIBRARY CONSTRUCTION AND SEQUENCING

Materials

- Ligase Buffer (NEB, cat. B0202S)
- dNTPs (NEB, 10 mM ea., cat. N0447L)
- T4 Polynucleotide Kinase (NEB, M0101L, 10,000 u/mL)
- T4 DNA Polymerase (NEB, M0203L, 3,000 u/mL)
- Klenow DNA Polymerase (NEB, Large fragment, 0.2 mL, 5000 u/mL, cat. M0210L)
- QIAGEN MinElute PCR purification kit
- NEB#2 10X Buffer (NEB, B7002S)
- dATP (NEB, 100 mM, N0440S)
- Klenow Fragment 3'→5' exo-(NEB, M0212L, 5,000 u/mL)
- RNase (Roche, DNase-free, 500 µg, 1 mL, cat. 11119915001)
- SPRI beads (Agencourt AMPure, Beckman Coulter, cat. APN 000130)
- T4 DNA Ligase (Enzymatics, L603-HC-L, 600 U/µL, 400 µL)
- PE adaptors (Illumina or same from IDT)
- PE PCR primers (Illumina or same from IDT)
- 2X Phusion HF Mastermix (Finzymes, P-531)
- SYBR Green I 10,000x concentrate in DMSO (Invitrogen, 500 µL, cat. S7563)
- ROX Passive Reverence Dye (USB, cat. 75768)
- Agilent 2100 Bioanalyzer
- High Sensitivity DNA Reagents for Agilent 2100 Bioanalyzer (cat. 5067-4627)
Applied Biosystems 7500 or 7500 Fast Real-Time PCR System

Protocol

1. Start with 100 μL of sheered CHART enriched nucleic acids (SECTION 6).

2. Add directly to the solution:
   - 13 μL Ligase Buffer (NEB)
   - 5 μL dNTPs (10 mM ea.)
   - 5 μL T4 PNK (NEB)
   - 5 μL T4 DNA Polymerase
   - 1 μL Klenow DNA polymerase (large fragment)

   Incubate 30 min. at 20°C in thermal cycler (rotate 1000 rpm)

3. Add 650 PB to each reaction and spin through a QIAGEN minElute column. Follow directions (rinse with 750 μL PE, spin 1 min, dry) and elute with 2x16 μL of EB.

4. To all 32 μL of the purified, end repaired material from 5, A-tail by adding:
   - 5 μL NEB#2 10X Buffer
   - 10 μL dATP (1 mM) NOTE: Stocks are usu. 100 mM.
   - 3 μL Klenow (exo-; NEB)

   Quick spin and incubate at 37°C in thermal cycler for 25 min.

5. Add 1 μL of RNase (DNase free, Roche) and continue incubation at 37°C for 5 min.

6. SPRI exchange using (1.8 vol, 90 μL) of SPRI beads as follows:

   - Warm SPRI beads to room temp
   - Add 90 μL of SPRI beads.
   - Vortex (at medium speed) and spin (10 sec at 1000 rpm).
   - Incubate 5 min at rt.
   - Capture 8 min on magnetic stand.
   - Remove supernatant carefully and discard.
   - Add 180 μL of freshly made 80% EtOH without disturbing pellet.
   - After ~30 sec., remove rinse.
   - Add another 180 μL of 80% EtOH as before.
   - After ~30 sec., remove rinse.
   - Quickspin (10 sec at 1000 rpm) and remove remaining sup.
   - Dry 5 min open at rt (covered with Kimwipe)
   - Resuspend beads in 9.0 μL of EB (from QIAGEN kit)
   - Vortex, let stand 2 min, vortex, spin (10 sec at 1000 rpm),
   - Capture for 1 min and transfer 8 μL of the purified DNA to a 1.7 mL tube.
7. To the 8 µL of purified, A-tailed DNA, set up the ligation reaction by adding:

12.5 µL 2X Ligation buffer (Enzymatics)
2 µL PE Adaptors (1 µM stocks)
2.5 µL T4 DNA Ligase (Enzymatics)

Incubate at room temperature for 15 min.

8. SPRI purify as per step 6 using 40 µL beads (1.6 vol) and elute with 15 µL EB.

9. Use 2.5 µL of the purified ligation to determine the number of PCR cycles necessary for amplification as follows:

Make a master mix (e.g., 5x) of the following for the correct number of rxns:
12.5 µL 2X Phusion (62.5 µL)
1 µL each primer (PE PCR primers 10 µM stocks) (5 µL)
0.5 µL 1:2000 SYBR green (2.5 µL)
0.25 µL ROX internal reference (1.25 µL)
7.25 µL H₂O (36.25 µL)

Add 22.5 µL/well of the qPCR plate

Then add 2.5 µL of the ligated library.

10. Analyze by qPCR with the following settings:

   Step 1: 98°C 30 sec.
   Step 2: 98°C 10 sec.
   65°C 20 sec.
   72°C 30 sec.
   repeat 20 cycles

   Volume: 25 µL

11. Pick the cycle number at the top of the linear phase of the amplification curve and use this value to perform a conventional PCR with the sample program using the following:

Make a master mix of the following for the correct number of rxns:

25 µL 2X Phusion MM
2 µL each primer (Illumina PE PCR primers, 10 µM stocks)
16 µL H₂O
Add 45 µL/tube of a PCR 8-strip

Then add 5 µL of the ligated library.

12. PCR with the following settings:

   Step 1: 98°C 30 sec.

   Step 2: 98°C 10 sec.
           65°C 20 sec.
           72°C 30 sec.
           repeat the number of cycles determined by qPCR

   Step 3: 72°C 5 min.

13. Purify with 80 µL SPRI beads eluting in 15 µL EB.


   Examine the size distribution of the library (should be around 200-500 bp with
   average fragment size 250-300 bp), the absence of lower molecular weight
   contaminants (peaks around 100-200 bp, possibly representing no insert
   products), and the concentration of the sample.

15. Save 1 µL of each library diluted into 50 µL H₂O for qPCR (SECTION 8).

16. Sequence this material on a next-generation sequencer.

**APENDIX 1. ANALYSIS OF CHART RNA ENRICHMENT BY qPCR**

**Materials**

PureLin Micro-to-Midi Total RNA Purification System (Invitrogen, cat. 12183-018)
VILO Reverse-transcription cDNA synthesis kit (Invitrogen, cat. 11754-050)
iTaq SYBR Green Supermix with ROX (Bio-Rad, cat. 172-5850)
ABI 7500 qPCR Instrument
Appropriate primer sets (see PRIMERS)

**Experiment**

1. Purify the CHART enriched, crosslink reversed RNA (SECTION 6, Step 3) and input
   sample as a control using a standard column purification (e.g., PureLink, Invitrogen).

2. Set up reverse transcription reactions as follows:
2 µL 5X VILO master mix
7 µL RNA solution from step 1.
1 µL VILO RT enzyme (include one RT-)

3. Incubate as instructed (25°C 10 min.; 42°C 60 min.; 85°C 5 min.; 4°C forever).

4. Dilute the RT reactions with ddH₂O (30 µL).

5. Analyze by qPCR using a ABI 7500 RT-PCR instrument and BIO-RAD iTaq SYBR Green Supermix with ROX (the dye, unrelated to roX2).

12.5 µL Supermix (need about 1.25 mL/plate)
7.5 µL of primer mix (3 µL ea. primer into 300 µL H₂O)
5 µL of RT reaction (use multichannel pipette to add and mix)
(94°C 5 min. 40 cycles of [94°C 30”, 52°C 30”, 72°C 1’]).

6. Calculate yields as follows:

\[
\text{Yield} = \left( \frac{\text{Input dilution factor}}{\text{efficiency}^{C_{T,\text{CHART}} - C_{T,\text{INPUT}}}} \right)
\]

The efficiencies for each primer set can be determined experimentally (the values should be ~2). Given the high yields of roX2 recovered by CHART, it is convenient to use an input that is diluted to 20% equivalents.

APENDIX 2. ANALYSIS OF CHART DNA ENRICHMENT BY qPCR

Materials

iTaq SYBR Green Supermix with Rox (Bio-Rad, cat. 172-5850)
ABI 7500 qPCR Instrument
Appropriate primer sets (see PRIMERS)

Experiment

CHART enrichment should be analyzed both before and after library construction by qPCR. Before library construction (from SECTION 6), the data is analyzed as yield relative to input:

\[
\text{Yield} = \left( \frac{\text{Input dilution factor}}{\text{efficiency}^{C_{T,\text{CHART}} - C_{T,\text{INPUT}}}} \right)
\]

After library construction, the diluted libraries (Step 15, SECTION 7) in triplicate using primers that will amplify known or expected binding sites (e.g., the endogenous roX2 locus and CES-5C2), and negative controls (e.g., Pka and Act-5C). Include a library constructed from the input. Note that the C_T values for the input
with different primers should be very similar to each other (within 1-2 \( C_T \) values). Normalize the signal to input and to one of the negative control (e.g., Act-5C, which was used because amplification of the PKA was undetected for all three replicates in the \( roX2 \) CHART enriched samples):

\[
\text{Fold enrichment} = \left( \frac{\text{efficiency}^{\frac{C_T,\text{CHART} - C_T,\text{INPUT}}{\text{TARGET PRIMERS}}}}{\text{efficiency}^{\frac{C_T,\text{CHART} - C_T,\text{INPUT}}{\text{ACT-5C PRIMERS}}}} \right)
\]

It is not rare that the CHART enriched library has undetectable levels of one of the negative controls. A conservative estimate of the enrichment can be made by entering \( C_T \) values of 40 in cases where no amplification is observed after 45 cycles.

**REAGENTS AND SOLUTIONS**

**Glycerol Buffer (500 mL)**
- 25% Glycerol (125 mL neat)
- 10 mM HEPES pH 7.5 (5 mL of 1 M)
- 1 mM EDTA (1 mL of 0.5 M)
- 0.1 mM EGTA (50 µL of 1 M)
- 100 mM KOAc (16.7 mL of 3 M stock)

Immediately before use, add to 40 mL:
- 0.5 mM Spermidine (200 µL of 0.1 M, aliquoted -80°C)
- 0.15 mM Spermine (60 µL of 0.1 M, aliquoted -80°C)
- 400 µL Complete EDTA-free Protease Inhibitor (from 100X stock)
- 1 mM DTT (40 µL of 1 M)
- 200 u SUPERasIN (20 µL of 20 u/µL)

**Sucrose Buffer (500 mL)**
- 0.3 M Sucrose (51.3 g solid)
- 1% Triton-X (50 mL of 10% Stock)
- 10 mM HEPES 7.5 (5 mL 1 M)
- 100 mM KOAc (16.7 mL 3 M stock)
- 0.1 mM EGTA (50 µL of 1 M)
- 100 mM KOAc (16.7 mL of 3 M stock)

Immediately before use, add to 20 mL:
- 0.5 mM Spermidine (100 µL of 0.1 M, aliquoted -80°C)
- 0.15 mM Spermine (30 µL of 0.1 M, aliquoted -80°C)
- 200 µL Complete EDTA-free Protease Inhibitor (from 100X stock)
- 1 mM DTT (20 µL of 1 M)
- 200 u SUPERasIN (10 µL of 20 u/µL)

**Nuclei Rinse Buffer (100 mL)**
- 50 mM HEPES pH 7.5 (5 mL 1M)
- 75 mM NaCl (1.5 mL 5M)
- 0.1 mM EGTA (20 µL of 0.5M)
Immediately before use dilute 0.5 mL into 4.5 mL H2O, add:

- 200 u SUPERasIN (5 µL of 20 u/µL)
- 1 mM DTT (5 µL of 1 M DTT)
- 50 µL of 100X protease inhibitors

**Sonication Buffer (10 mL)**

- 50 mM HEPES pH 7.5 (500 µL 1 M)
- 75 mM NaCl (150 µL 5 M)
- 0.1 mM EGTA (2 µL of 0.5 M)
- 0.5% N-Lauroylsarcosine (1 mL, 5%)
- 0.1% Sodium deoxycholate (100 µL, 10%)

Immediately before use add (to 5 mL):

- 100 u SUPERasIN (5 µL of 20 u/µL)
- 5 mM DTT (25 µL of 1 M DTT)

**Wash Buffer 100 (50 mL)**

- 100 mM NaCl (1 mL of 5 M stock)
- 10 mM HEPES pH 7.5 (500 µL of 1 M)
- 2 mM EDTA (200 µL of 0.5 M stock)
- 1 mM EGTA (100 µL of 0.5 M stock)
- 0.2% SDS (1 mL of 10% stock)
- 0.1% N-lauroylsarcosine (1 mL of 5% stock)

Immediately before use:

- Add 100 µL PMSF (0.4 mM stock)
- Filter (0.22 µm)

**Wash Buffer 250 (50 mL)**

Same as WB100 except with 250 mM NaCl.

**Denaturant Buffer**

- 8 M urea
- 200 mM NaCl
- 100 mM HEPES pH 7.5
- 2% SDS

**2X Hybridization Buffer**

- 1.5 M NaCl
- 1.12 M Urea
- 10X Denhardt’s Solution
- 10 mM EDTA

**Biotin Elution Buffer**

- 12.5 mM Biotin solution (25 µL of 50 mM stock)
dilute with WB250 (75 µL)

**RNase H-elution Buffer (HEB, 2 mL)**
- 50 mM HEPES pH 7.5 (100 µL 1M)
- 75 mM NaCl (30 µL 5M)
- 0.125% N-Lauroylsarcosine (0.1 mL, 5%)
- 0.025% Sodium deoxycholate (4 µL, 10%)
- 40 u SUPERasIN (2 µL of 20 u/µL)
- 10 mM DTT (20 µL of 1 M DTT)

**Nucleic Acid XLR Buffer (400 µL)**
- 100 µL Tris 7.5 (1M Stock)
- 100 µL SDS (10% Stock)
- 200 µL Proteinase K solution (20 mg/mL)